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(54) Title: ACOUSTICALLY REFLECTIVE LIPOSOMES AND METHODS TO MAKE AND USE THE SAME (57) Abstract This invention relates to tissue specific acoustically reflective oligolamellar liposomes containing internally separated bi-layers and methods to make and to use the same, alone as a perfusion ultrasonic contrast agent or conjugated to a ligand for tissue-specific ultrasonic image enhancement.		

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**Acoustically Reflective Liposomes and Methods
to Make and Use the Same**

1 Background of the Invention:

Field of this invention: This invention relates to acoustically reflective oligolamellar liposomes containing internally separated bilayers and methods to make same for
5 ultrasonic image enhancement.

Description of the Prior Work in the Field: Ultrasonic assessment of internal body organs or tissue is a well known technique. This assessment can be made by sending a signal with a waveform in the high frequency sound
10 spectrum (10^6 cycles/second) and detecting the reflected ultrasound properties. In current ultrasound procedures, the inherent reflective properties of the tissue are studied.

In one recent patent, U.S. Patent No. 4,900,540 a
15 method for producing liposomes having an encapsulated gas is described. Liposomes are phospholipid bilayers discretely encapsulating an aqueous compartment. The composition and form of these lipid vesicles are analogous to that of cell membranes with hydrophilic polar groups
20 directed inward and outward toward the aqueous media and hydrophobic fatty acids intercalated within the bilayer. Liposomes form spontaneously from a dry lipid film exposed to an aqueous medium and may be unilamellar and/or multilamellar. Unilamellar vesicles are typically

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1 classified as small (20 to 200 nm diameter) or large
(approximately 1 micron). Multilamellar liposomes are
classically described as having concentric bilayers, an
"onion morphology". A type of multilamellar liposome
5 termed oligolamellar liposomes are typically described as
multilamellar liposomes with increased aqueous space
between bilayers or have liposomes nested within bilayers
in a nonconcentric fashion. Liposomes have many uses but
10 are considered to be highly desirable for drug delivery
and diagnostic applications.

As previously discussed, the "540" patent discloses a
method for producing liposomes having an encapsulated
gas. It is said that these liposomes can be injected for
15 in vivo enhancement of organ imaging with ultrasound. A
gas charged particle in vivo, however, may not be stable.
Thus, it is desirable to develop a stable acoustically
reflective liposome by controlling composition, structure
and size alone.

20

Summary of the Invention

This invention provides acoustically reflective
liposomes amenable to ligand conjugation for targeted
ultrasonic enhancement. These liposomes enhance the

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1 acoustic reflectivity based solely on their composition,
structure and size. This invention provides an
acoustically reflective oligolamellar liposome containing
internally separated lipid bilayers. More specifically,
5 this invention provides an acoustically reflective
liposome particle made by incorporating
phosphatidylethanolamine with other lipids and having a
mean particle size of between about .8 and 10 microns.
This composition has a oligolamellar structure and it is
10 theorized that the resultant lamellar structure together
with the mechanical properties of the phospholipid bilayer
make the liposomes acoustically reflective. The liposomes
typically include phospholipids such as
phosphatidylcholine and phosphatidylethanolamine and can
15 also include cholesterol.

This invention further provides a composition wherein
the acoustically reflective liposome may be conjugated to
a tissue specific ligand. Preferably, this ligand is an
antibody or antibody fragment.

20 The acoustically reflective liposome of this invention
can be made by incorporating phosphatidylethanolamine into
a typical phosphatidylcholine based liposome particle,
optionally reducing the size of the particle to less than
about 400 nm, lyophilizing the particle, and reducing the

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1 particle size to between about 0.8 and 10 microns. This
liposome particle can be chemically altered to covalently
couple a tissue specific ligand. As an alternative to
lyophilization, the acoustically reflective liposomes of
5 this invention can be made by double-emulsion method in an
organic solvent such as triolein or by repeated
freeze-thawing of the liposomes. This invention further
provides a method to prepare targeted, acoustically
reflective liposomes to characterize specific tissues
10 involving administering ligand-conjugated acoustically
reflective, oligolamellar liposomes containing internally
separated bilayers devoid of gas, and measuring ultrasonic
reflectance of the specific tissue of interest before and
after liposome administration and comparing the results
15 for enhanced acoustic reflectance. Specifically, this
method can be used to characterize a ventricular thrombus;
although many other types of tissue can be analyzed. This
invention also provides a method to enhance tissue
perfusion (i.e. use as a contrast agent). In these
20 situations, the contrast agent could be the liposome
without conjugated ligands.

This invention also provides a method to monitor a
drug delivered in a liposome administered to a patient's
circulatory system. This method involves mixing a
25 liposome containing the drug targeted to specific tissue
and (if the drug containing liposome is not acoustically

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- 1 reflective) an acoustically reflective liposome devoid of
any gas targeted to the same tissue to form a dispersion,
administering the dispersion to a patient and monitoring
the delivery of the liposome containing the drug by
5 detecting the acoustically reflective liposome.

Brief Description of the Figures

Figure 1 is an ultrasonic image of a buffer control
obtained in vitro using 20 MHz intravascular catheter.

- 10 Figure 2 is an ultrasonic image of pure
phosphatidylcholine vesicles prior to extrusion through
NUCLEPORE (Costar Co.) membranes obtained in vitro using
20 MHz intravascular catheter.

- Figure 3 is an ultrasonic image of phosphatidylcholine
15 and cholesterol vesicles prior to extrusion obtained in
vitro using 20 MHz intravascular catheter.

Figure 4 is an ultrasonic image of phosphatidylcholine
and phosphatidylethanolamine vesicles prior to extrusion
in vitro using 20 MHz intravascular catheter.

- 20 Figure 5 is an ultrasonic image of
phosphatidylcholine, phosphatidylethanolamine and
cholesterol vesicles prior to extrusion in vitro using 20
MHz intravascular catheter.

- Figure 6 is an ultrasonic image of a
25 phosphatidylcholine and cholesterol vesicles after

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1 extrusion in vitro using 20 MHz intravascular catheter.

Figure 7 is an ultrasonic image of MHz intravascular catheter in vitro phosphatidylcholine and phosphatidylethanolamine vesicles after extrusion obtained
5 in vitro using 20 MHz intravascular catheter.

Figure 8 is an ultrasonic image of phosphatidylcholine, phosphatidylethanolamine and cholesterol vesicles after extrusion obtained in vitro using 20 MHz intravascular catheter.

10 Figure 9 is a schematic drawing of different types of liposome particles, A-E.

Detailed Description of the Invention and Best Mode

In accordance with this invention, acoustically
15 reflective liposomes are provided which may be used alone as an ultrasonic contrast agent or which are conducive to site specific ligand conjugation to enhance ultrasonic imaging of specific tissues types or receptors in the body.

20 The inventors have discovered that the echogenicity of liposomes is a function of composition, morphology and size. With respect to morphology, the inventors have found that liposomes may be simply produced by mechanical dispersion of a dried phospholipid film (e.g.
25 phosphatidylcholine) into an aqueous medium. This procedure typically produces classical multilamellar and

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1 fewer oligolamellar vesicles. The addition of
phosphatidylethanolamine imparts morphological changes to
the arrangement of the bilayers. It is hypothesized that
the head groups of the phosphatidylethanolamine results in
5 this acoustically reflective arrangement, speculated to be
an oligolamellar type of liposome. Furthermore, liposomes
with similar acoustic properties may be produced by the
inclusion of charged lipids, (e.g. phosphatidylserine)
which could lead to a more oligolamellar distribution
10 secondary to internal repulsion of the lipid bilayers.

More specifically, the above described oligolamellar
liposomes possess a series of membrane-fluid interfaces
that in the aggregate are ultrasonically reflective.
Liposomes produced by the same method, but without the
15 incorporation of phosphatidylethanolamine or charged
lipids (e.g. pure phosphatidylcholine vesicles) are not
echogenic because, despite their multilamellar morphology,
the bilayers are typically closely opposed and act as a
single acoustic interface.

20 Although both classic multilamellar and oligolamellar
liposomes may be created directly from a dried lipid film
without lyophilization, the inventors suspect that the
classical multilamellar form predominates this approach
and that such vesicles are less echogenic than the
25 oligolamellar form which may predominate after rehydration

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1 of freeze-dried liposomes. See Figure 9. Figure 9A
demonstrates a small unilamellar liposome, Figure 9B a
large unilamellar liposome, Figure 9C a classical
5 multilamellar liposome (classical), Fig. 9D and 9E two
types of oligolamellar liposomes (concentric and vesicular
respectively) Liposomes 9A, 9B and 9C are nonechogenic,
i.e. minimally acoustically reflective. It is believed
that liposomes 9A, 9B and 9C do not have a sufficient
10 number of interfaces for detectable acoustic reflection.
Liposomes 9D and 9E are echogenic, i.e. acoustically
reflective, probably due to the bilayers being separate
enough to give sufficient reflective interfaces.

Ultrasonically reflective liposomes may be created by
any procedure which creates oligolamellar vesicles with
15 internally separated bilayers. The inventors have
demonstrated the use of liposome composition (i.e.
phosphatidylethanolamine incorporation) to create this
effect, but one can easily envision mechanical means to
the same end. One example is the conjugation of ligands
20 (i.e. antibodies, peptides, lectins, etc.) to lipid
components of the membrane then incorporation of these
components between layers of the multilamellar vesicles by
a suitable process. In this scenario, the relatively
large size of the ligands bound to the inner and outer
25 bilayer surfaces could either primarily spread or

1 secondarily enhance the separation of multilamellar bilayers.

oligolamellar liposomes may be prepared by but are not limited to the following processes: lyophilization, repeated freeze-thaw, a modified double emulsion technique. Production through lyophilization is the current standard operating procedure. The acoustically reflective liposome particle is made by combining phosphatidylethanolamine with other lipids (e.g. phosphatidylcholine) into a dried film, resuspending the film with deionized water with or without cryoprotectant, to form a liposome, reducing the size of the particles to less than about 400 nm, lyophilizing the particles, and resuspending the particles in buffer. Particles may then be extruded to a size between about 0.8 and 10 microns. This method is the most conducive to conjugating protein ligands since it avoids exposure of the protein to organic solvents, evaporation or mechanical destruction. The antibody is conjugated in an aqueous buffer to unilamellar liposomes less than 400 nm in diameter for maximum efficiency and the conjugate may be freeze-dried with mannitol to help protect the peptide while allowing the vesicles to structurally degrade.

Alternatively, in the modified double emulsion method,
25 an organic solution containing triolein, phospholipids and

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1 cholesterol is combined on a volume to volume basis with
an aqueous solution typically containing materials to be
entrapped. This solution is vigorously agitated to form a
water-in-oil emulsion. This emulsion is then combined
5 with a sucrose solution and agitated again, creating a
water-in-oil-in-water emulsion, or a double emulsion.
Evaporation of the organic solvent leaves
multicompartmental liposomes. This method is compatible
with liposome encapsulation of but is less conducive for
10 conjugate with proteins or other biological macromolecules
(Kim, S., Turker, M.S., Chi, E. Y., Sela, S. and Martin,
G.M., 1983 Biochem. Biophys. Acta 728, 339; Gao, K. and
Huang, L., 1987 Biochem. Biophys. Acta 897, 377).

Another method for producing oligolamellar liposomes
15 is a variant of the lyophilization method and involves
freezing and thawing of small unilamellar liposomes (Pick,
U. 1981 Arch. Biochem. Biophys., 212, 186). The repeated
freeze-thawing of liposomes leads to membrane rupture and
reannealing into larger multicompartmental vesicles.
20 These membranes typically incorporate charged
phospholipids into the bilayers to provide a nidus for ice
crystal formation. Although the protein ligands could
easily be conjugated to the smaller unilamellar liposomes,
repeated freeze-thawing of biologically active proteins
25 may destroy their bioactivity and reduce their efficacy as
targeting ligands.

1 The liposomes which have been found to be useful in
this invention incorporate phosphatidylethanolamine into
the bilayer liposomes that can be formed into a vesicular
structure. The liposome can be formed from lipids such as
5 phosphatidylcholine (PC) and phosphatidylethanolamine
(PE). Preferably the phosphatidylcholine ranges from
about 50 to 95 mol % of the lipid content of the liposome,
but can generally range from 60 to 90 mol % of the
liposome, while phosphatidylethanolamine preferably ranges
10 from 2 to 20 mol %, but generally from about 5 to 10 mol
percent of the lipid content of the liposome.

Incorporation of cholesterol, another neutral lipid,
has been found to contribute an echogenic component to
liposomes which was lost after polycarbonate extrusion,
15 suggesting that large liposome size increases
echogenicity. Work by Rhoden and Golden (Rhoden, V and
Goldin, S 1979 Biochemistry 18, 4173) has indicated that
incorporating cholesterol into the lipid bilayer will
increase particle size while, the addition of charged
20 phospholipids decrease vesicle size. Cholesterol
intercalates within the phosphatidylcholine bilayer with
very little change in area by occupying the regions
created by the bulky phosphatidylcholine headgroups. This
increases the packing density and structural stability of
25 the bilayer and may contribute to acoustic character (New,

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- 1 R.R.C., 1990 In New, R.R.C. (ed): Liposomes: a practical approach, (ed), Oxford University Press, New York, pp 19-21).

5 With respect to size, it was found that liposomes of approximately one to four microns in size were lyophilized without excipients and were echogenic upon resuspension with Tris buffer, pH 7.5. Additionally, it was found that decrease of prelyophilization particle size to less than one micron with probe sonication and a sugar excipient
10 resulted in echogenic liposomes. During lyophilization, the smaller vesicles may break down more completely and enhance the lipid interactions upon rehydration. The addition of sugar (mannitol) was selected to provide bulk to the lyophilized powder without cryoprotecting the
15 vesicle size. Other sugars, such as trehalose or maltose can be used to provide bulk, but also tend to preserve liposome integrity during freeze-drying and may be contraindicated in the production of echogenic liposomes from small unilamellar liposomes.

20 The smaller the liposomes the higher the ultrasonic frequency required to resolve the vesicle. In practice, a single liposome (1-2 microns) is not resolvable with commercially available diagnostic ultrasonic transducers. However, discrete liposomes and the coalescence of
25 liposomes either randomly in solution or secondary to

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- 1 specific targeting ligands contribute to ultrasonic
scattering, and can create an acoustic interface which may
be visualized and quantified. Moreover, in vivo, targeted
echogenic liposomes will benefit from enhanced
5 echogenicity secondary to creation of multiple
liposome-tissue interfaces and a transient increase in
target tissue size and density.

An acoustically reflective liposome particle generally
ranges from .8 to 10 microns, but preferably 1.0 to 3.0
10 microns.

In summary, the lyophilization method (referred to as
dehydration-rehydration procedure) appears to provide the
preferred method for creating echogenic, protein-targeted
liposomes. The small unilamellar vesicles are optimum for
15 protein conjugation and can be ruptured and enlarged into
multivesicular liposomes without destroying the biological
activity of the ligand. This method was first suggested
by Kirby and Gregoriadis (Kirby, C. and Gregoriadis, G.,
1984 Biotechnology 2, 979 (hereby incorporated by
20 reference) for the purpose of increasing liposome
entrapment efficiency. The inventors have discovered that
liposomes produced by this method with the appropriate
chemical composition are echogenic and suitable for
targeting.

25 The acoustically reflective liposomes can be
conjugated to a site specific ligand such as an antibody,

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- 1 lectin, peptide or nucleic acid. A variety of methods
have been reported to attach proteins to liposomes
covalently. (Martin, F.J., Heath, T.D. and New, R.R.C.,
1990 In Liposomes: a practical approach. Oxford
5 University Press, New York, pp 163-182; hereby
incorporated by reference). The most popular methods
involve synthesis of thiol-reactive lipids using either
N-succinimidyl-pyridyl-dithiopropionate (SPDP) or
N-succinimidyl-(4-[p-maleimidophenyl])-butyrate (SMPB).
10 SPDP produces a reversible disulfide bond and SMPB
produces an irreversible thioester. The conjugation
process is essentially the same for both reagents and the
inventors have elected to use SPDP for in vitro
experiments reported herein.
- 15 S-acetylmercaptosuccinic anhydride can be used instead
of SPDP with a substitution of hydroxylamine for
dithiothreitol in the process to reduce the number of
purification steps required (Martin, F.J., et al. Supra.
163-182).
- 20 Another method described by Heath et al. (Heath, T.D.,
Maher, B.A. and Paphadjopoulos, D., 1981: Biochem.
Biophys. Acta 599, 42) involves the use of periodate to
create a Schiff base between glycolipids incorporated into
the lipid membrane and primary or secondary amino groups
25 on proteins. This method provides 20% binding of the

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- 1 initial protein with a theoretical maximum of 40%.
- Conjugation to larger liposomes appears better than smaller unilamellar liposomes, a factor which may be less desirable for producing echogenic liposomes by
- 5 lyophilization.

Phosphatidylethanolamine can also be derivatized by attaching a bifunctional straight-chain (6-8 carbons) dicarboxylic acid which can bridge between the lipid and protein. The phosphatidylethanolamine may be derivatized

10 by either a di-N-hydroxysuccinimide derivative or by reaction with carbodiimide. The former route must be used with a process which prepares liposomes rapidly without significant subsequent processing. The carbodiimide method is prone to extensive cross-linking and often

15 requires citraconylate blocking of endogenous amino groups. This blocking reagent is removed at pH 4.4 which may precipitate peptides or begin to hydrolyze the liposome membranes (Martin, F.J., et al. Supra. at 163-182).

- 20 Liposomes can be conjugated with Protein A or Protein G, which have inherent affinity for immunoglobulins. Liposomes can be conjugated with avidin, which strongly binds to biotin that can be attached to immunoglobulins or other ligands thereby effecting a coupling of the ligand
- 25 to liposomes. Finally, sugars and other oligosaccharides

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1 may be conjugated to liposomes containing a pure or
SPDP-derivatized phosphatidylethanolamine via endogenous
aldehyde groups of the saccharide by introducing a thiol
residue onto the sugar or by carboxylating free hydroxyl
5 groups with succinic anhydride followed by a carbodiimide
coupling reaction. (Martin, F.J., et al. Supra at 163-182).

An acoustically reflective liposome particle may be
used alone as an ultrasonic contrast agent or with a
ligand conjugation for specifically targeted ultrasonic
10 image enhancement. More specifically, ligand-targeted,
acoustically reflective liposomes may be used to enhance
ultrasonic imaging of intravascular structures as well as
extravascular structures accessible due to increased
permeability of the vasculature or by direct
15 administration into a nonvascular space. The potential
cardiovascular targets include myocardial tissue
(antimyosin antibody), vascular clot (anti-fibrin
antibody), vegetations (anti-bacterial determinant
antibody), endothelial surface (anti-receptor or surface
20 determinant antibody) and tumors (anti-tumor antibody).
Additionally, other tissue structures in the penetrable
spaces of joints spaces, (lymphatic system; urogenital or
pulmonary bronchial alveolar tree) may be ultrasonically
enhanced with specific acoustically reflective liposomes
25 administered directly into these spaces.

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1 The routes of administration include intravascularly,
intralymphatically, intrathecally, intraurologically,
intracapsularly, and bronchial lavage. The acoustically
reflective liposome made be administered as a bolus of
5 liposomes or as an infusion in a pharmaceutically
acceptable carrier such as saline or glucose in water.

As with other injected pharmaceutical agents, the
acoustically reflective liposome, is administered as a
sterile compound. To prepare a sterile composition of
10 matter the acoustically reflective liposome is prepared
under aseptic conditions using sterilized buffers and
reagents.

Visualization of the in situ liposomes is possible
with virtually all ultrasonic imaging modalities,
15 including intravascular (catheter-based), transcutaneous
transvascular/epicardial (conventional echocardiography,
vascular or high frequency) and transesophageal
echocardiography. The precise imaging technique
appropriate for a given application must take into account
20 the clinical objective of the procedure, anatomic site of
interest and medical condition of the patient.

Two important uses for liposome enhancement to tissue
would be the following. First, the standard
transcutaneous or transesophageal ultrasound would be used
25 to identify cardiac structure. Liposomes would

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1 subsequently be used to enhance structure definition,
either through their perfusion into a vascular bed to
identify regions of perfusion (i.e. myocardial) or to
identify pathologic structures by directly or indirectly
5 "highlighting" of the target. The transducers that are
used utilize probes from 2 to 15 MHz and are placed
transcutaneously/transvascular/epicardially/or
transesophageally to image cardiovascular structures.

In the second instance, liposomes would be utilized in
10 conjunction with intravascular ultrasound imaging
devices. These devices generate images of vascular
structure and operate at a frequency of 10-100 MHz. These
catheters do not generally impede blood flow and they
would be directed to the region of interest with the
15 liposomes highlighting structures such as plaque, thrombus
or endothelial receptors/determinants.

Using either the intravascular or the
transcutaneous/transvascular/epicardial/transesophageal
method, the 2-dimensional B-mode ultrasound images
20 generated are amenable to more sophisticated image
processing and/or analysis. Gray-scale texture analysis,
radiofrequency signature analysis and a variety of
additional complementary ultrasonic material
characterization techniques may find use in enhancing the
25 interpretation of these data.

EXAMPLE 1Production of Echogenic Liposomes Using a
Lyophilization Methodology "Best Mode"

- 1 This procedure describes methods and reagents required to make acoustically reflective, oligolamellar liposomes using a composition and method conducive to the production of antibody targeted vesicles. Freeze-drying is
- 5 lyophilization

Reagents:

- 1) Phosphatidylcholine, from egg yolk (PC) as an alcoholic solution
- 2) Cholesterol (Chol)
- 10 3) Phosphatidylethanolamine, dipalmitoyl (PE)
- 4) Deionized water
- 5) Cryoprotectant sugar (e.g. mannitol)
- 6) Tris HCl
- 7) Sodium Phosphate (dibasic)
- 15 8) Sodium Chloride

Equipment:

- 1) Rotary evaporator with external cooling, vacuum and heating.
- 2) Probe sonicator and ear protection.
- 20 3) Submicron particle size analyzer (e.g. NICOMP (Pacific Scientific) Malvern) and tubes.

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- 1 4) Volumetric flasks, graduated cylinders, syringes,
hypodermic needles, repipets/pipets and liquid
scintillation vials (25 & 10 mL).
- 5 5) Stirrer/heating plate and stir bars.
- 6) Electronic balance (accurate to 0.1 or 0.01 mg).
- 7) pH meter and calibration standards.
- 8) Ring stand and clamps.
- 9) NUCLEOPORE (Costar Co.) filters and membranes in steps
from 2 to 0.22 microns.
- 10 10) Bottle lyophilizer and associated equipment.
- 11) CVIS intravascular catheter system and 20 MHz imaging
catheter.

Procedure:

- 1) Warm vials of phosphatidylcholine and
15 phosphatidylethanolamine to room temperature.
- 2) Weigh (according to electronic scale instructions)
25mg of cholesterol and 20 mg of
phosphatidylethanolamine.
- 3) Dissolve cholesterol in 2.5 mL of dry chloroform and
20 phosphatidylethanolamine in 2.0 ml of dry chloroform
using supplemental heat as required.
- 4) Combine in a 250 mL rotoevaporator flask: 73 mg of
phosphatidylcholine, 11 mg phosphatidylethanolamine
and 16 mg of cholesterol. Adjust as required for
25 scale-up.

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- 1 5) Attach flask to pre-cooled (0 degrees Centigrade)
rotary evaporator with a 50 degree Centigrade warming
bath.
- 5 6) Rotate flask rapidly (approximately 210 rpm) and dry
under vacuum.
- 7) After the film is dry, remove flask from
rotoevaporator and place in desiccator under vacuum
and cover desiccator with a dark cloth.
- 8) Continue to dry film under vacuum for 2 days.
- 10 9) Prepare 100 mM mannitol solution in deionized water.
- 10) Add 10 mL of mannitol solution per 100 mg of lipid.
- 11) Without significant agitation, place flask on
rotoevaporator without coolant circulating, flood
atmosphere with nitrogen and rotate the flask
15 (approximately 210 rpm) to resuspend lipid film.
- 12) When lipid film is resuspended, add one or two drops
of liposome suspension to NICOMP (Pacific Scientific)
tube and dilute with 100 mM mannitol solution,
sufficiently for analysis.
- 20 13) Analyze particle size with NICOMP (Pacific Scientific)
according to manufacturer's instructions.
- 14) Transfer liposomes from round bottomed flask to 25 mL
liquid scintillation vial with a pipet and cap.
- 15) Secure vial to ring stand and submerge vial
25 approximately two-thirds into a cool tap-water bath to
dissipate excess heat during sonication.

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- 1 16) Remove cap and position sonicator probe into the vial
being sure not to leave the probe in contact with the
glass and maintaining the tip of the probe about one
quarter inch above the bottom.
- 5 17) Set the sonicator for 10% duty and 10% power and
sonicate for 3 minutes.
- 18) Recheck particle size as before with NICO MP (Pacific
Scientific). Continue to reduce particle size using 1
minute sonication bursts at same power and with
10 increasing duty levels until vesicles are less than
400 nm. Increase power and repeat duty changes
starting at 10% if additional sonication is required.
- 19) Transfer approximately 5 mL aliquots of liposome
suspension into each of two 25 mL liquid scintillation
15 vials, layer with nitrogen, and cap.
- 20) Either snap-freeze in acetone-alcohol-dry ice bath or
freeze overnight in -70 Centigrade deep freezer.
- 21) Transfer vials from the freezer to the lyophilizer
jars and freeze dry according to manufacturer's
20 instructions for approximately 48 hours.
- 22) After 48 hours of lyophilization, remove vials from
the freeze dryer, gently overlay contents with
nitrogen, recap and seal vials with parafilm.
- 23) Store vials in refrigerator with desiccant up to 72
25 hours until use.

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- 1 24) Prepare 0.10 M Tris HCl + 0.10 M NaCl, pH 7.5 buffer.
- 25) To rehydrate, add 0.10 mM Tris HCl + 0.10 mM NaCl, pH
7.5, buffer to each vial. A good starting volume is
2-3 mL of buffer/mL of the desired liposome
5 lyophilized.
- 26) Size liposomes with the NICOMP (Pacific Scientific)
submicron particle analyzer as previously discussed.
- 27) Transfer liposome suspension to an appropriately sized
syringe and extrude liposomes to desired particle size
10 through polycarbonate membranes according to
manufacturer's instructions until desired size is
attained. Excessive extrusion will significantly
destroy liposome structure and diminish echogenicity.
If initial particle size is more than 1-2 microns
15 above desired range, repeat procedure using a greater
post-lyophilization dilution factor to attain a
smaller initial vesicle size. Be sure sample for size
analysis dilution buffer is iso-osmotic with liposome
solution (300 milliosmoles) to avoid artifactual
20 swelling or contraction of vesicles and inaccurate
size estimation.
- 28) Transfer liposomes into small (10 mL) liquid
scintillation vials and image liposome suspension with
20 MHz CVIS intravascular imaging catheter system.
- 25

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1. Reference: New, R.R., 1990, Liposomes: A Practical Approach. Oxford University Press. (hereby incorporated by reference).

EXAMPLE 2 .

- 5 A. SPDP Derivatization of Phosphatidylethanolamine
- The following procedures described methods to produce an N-succinimidyl pyridyl dithiopropionate (SPDP) derivitization of IgG antibody and phosphatidylethanolamine and their incorporation into a process for producing acoustically reflective liposomes as described in Example 1. This procedure describes methods and reagents required to make N-succinimidyl pyridyl dithiopropionate derivatives of phosphatidylethanolamine for incorporation into liposomes for antibody coupling
- 10 reaction.

Reagents:

- 1) L- α -phosphatidylethanolamine, dimyristoyl or dipalmitoyl, 99% purity (PE)
- 2) Triethylamine (TEA)
- 20 3) N-succinimidyl pyridyl dithiopropionate
- 4) Nitrogen
- 5) Silicic Acid
- 6) Methanol
- 7) Chloroform
- 25 8) Dodecamolybdophosphoric acid
- 9) Silica thin layer chromatography (TLC) plates

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1

Equipment:

- 1) Liquid scintillation vial or test tube with cap
- 2) Stirrer/heating plate and stir bars
- 5 3) 20cc plastic syringe barrel with glass fiber plug
- 4) Fraction collector or test tube rack
- 5) TLC developing tank with filter paper lining
- 6) Electronic balance (accurate to 0.1 or 0.01 mg)
- 7) Graduated cylinders, repipets/pipets
- 10 8) Ring stand and clamps
- 9) Atomizer sprayer and spray box
- 10) Rotary evaporator with external cooling, vacuum, heating and 250 mL flasks.
- 11) Hamilton microliter syringe with lock

15

Procedure:

- 1) Weigh 15 mg of PE, 10 mg of SPDP, 2 g silicic acid, 3 g dodecamolybdophosphoric acid.
- 2) Dissolve PE in a liquid scintillation vial in
20 approximately 2 mL chloroform using sufficient warming to facilitate dissolution but avoiding excess heat.
- 3) Dry PE under continuous nitrogen stream or with rotoevaporation.
- 4) Resuspend PE in 2.0 mL of chloroform.
- 25 5) Dissolve 200 microliters TEA in 25 mL methanol (2.9 mg/0.5 mL).

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- 1 6) Dissolve 10 mg SPDP in 0.5 mL methanol with slight supplemental heating.
- 7) Add 0.5 mL TEA (2.9 mg) to PE in vial.
- 8) Add 0.5 mL SPDP (10 mg) to PE and TEA.
- 5 9) Add small stirring bar to vial.
- 10) Saturate atmosphere in vial with nitrogen and cap.
- 11) Stir mixture at room temperature for 2 hours.
- 12) In a 250 mL or greater flask add 130 mL of chloroform, 50 ml of methanol, and 10 ml of deionized water.
- 10 13) If solution is cloudy add 1-2 mL additional methanol to dry.
- 14) Add approximately half of the solution to the developing tank.
- 15) Cover tank and allow atmosphere and filter paper lining to saturate with solvents.
- 15 16) Slurry 2 g of silicic acid in 10-12 mL of chloroform.
- 17) Pour silicic acid slurry into syringe barrel supported on a ring stand.
- 18) Allow excess solvent to drain but do not allow column to dry.
- 20 19) Prepare phosphomolybdate spray by dissolving 3 g of dodecamolybdophosphoric acid in 10 mL of ethanol or reagent grade alcohol.
- 20 20) Place in sprayer and wrap sprayer in foil to blockout light until use.
- 25

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- 1 21) After 2 hours check reaction by spotting (10
microliters) the reaction mixture and pure
phosphatidylethanolamine standards with a Hamilton
syringe onto the thin layer chromatography plate.
- 5 22) When the solvent line on TLC plate is approximately
1.0 cm from the top, remove the plate from the tank
and air dry.
- 23) In a hood, spray dry plate with a light, even coat of
phosphomolybdate spray.
- 10 24) Warm plate on a hot plate with low heat to develop
black spots (100 degrees Centigrade). The test is
qualitative. Derivatized phosphatidylethanolamine
migrates faster than the pure standard lipid. The
reaction is usually 100% complete. If the reaction is
15 incomplete, add 1 mg of TEA and allow reaction to
continue 30-60 minutes and recheck with thin layer
chromatography.
- 25) When the reaction is complete, add mixture to silicic
acid column and wash in with 4.0 mL chloroform.
- 20 26) Elute derivatized phosphatidylethanolamine in 2.0 mL
fractions using 4.0 mL aliquots of the following
chloroform:methanol solutions gradient: 4.0:0.25,
4.0:0.50 4.0:0.75, 4.0:1.0, 4.0:1.25, 4.0:1.5,
4.0:1.75, 4.0:2.0. Repeat 4.0:2.0 to ensure complete
25 elution.

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- 1 27) Layer each fraction with nitrogen after collection to inhibit oxidation.
- 28) Identify fractions with phosphatidylethanolamine derivative by thin layer chromatography as before.
- 5 29) Pool fractions containing derivative into a rotoevaporator flask.
- 30) Attach flask to rotoevaporator previously cooled with circulating cold water or coolant.
- 31) Dry derivative to a film and resuspend in 3.0 cc of
10 chloroform.
- 32) With thin layer chromatography roughly quantitate phosphatidylethanolamine derivative concentration by comparing 15 μ L, 10 μ L, and 5 μ L spots of
15 standard PE of known concentration to equal volumes of the derivative solution. Determine mg/mL of derivative.

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**B. N-Succinimidyl Pyridyl Dithiopropionate
Derivitization of IgG Antibody**

1 This procedure describes the N-succinimidyl pyridyl dithiopropionate derivitization (SPDP) of IgG antibody for conjugation with similarly modified liposomes.

5 Reagents:

- 1) N-hydroxysuccinimidyl-dithiopropionate (SPDP)
- 2) Sodium citrate
- 3) Anhydrous sodium phosphate, dibasic
- 4) Sodium chloride
- 10 5) Sephadex G-50
- 6) Ethanol or reagent grade alcohol
- 7) IgG antibody
- 8) BCA protein binding dye concentrate (BCA)
- 9) Dithiothreitol
- 15 10) Deionized water
- 11) Sodium acetate
- 12) Nitrogen
- 13) Dilute hydrochloric acid (HCl)
- 14) Glacial acetic acid (17.4 M)
- 20 15) Anhydrous potassium phosphate, monobasic

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1 Equipment:

- 1) UV/VIS Spectrophotometer
- 2) 100 mL Gel Chromatography column and tubing
- 3) Fraction collector
- 5 4) Micropipets
- 5) Hamilton syringe (5-10 μ L capacity)
- 6) Pasteur pipets
- 7) Beaker/flasks various sizes
- 8) Stirrer and stir bars
- 10 9) Analytical balance (accurate to 0.1 or 0.01 mg)
- 10) pH meter with micro and regular electrodes and appropriate standards
- 11) Ring stand and clamps
- 12) Amicon ultrafiltration system
- 15 13) Volumetric flasks

Procedure:

- 1) Combine 19.7 g sodium citrate, 7.1 g anhydrous sodium phosphate, dibasic (Na_2HPO_4), and 2.9 g sodium chloride per liter in a 1 or 2 liter beaker and fill
20 75% with highly purified, deionized water. Usually prepare 2 L of buffer.
- 2) Adjust pH to 7.0 using dilute hydrochloric acid.
- 3) Transfer pH 7.0 buffer to volumetric flask with three
25 washes of the beaker and fill to the mark with deionized water.

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- 1 4) Weight 7.5g of SEPHADEX G-50 (Pharmacia Co.) and
transfer to a 125 mL beaker.
- 5 5) Add 100 mL of citrate/phosphate buffer (step 1) to the
beaker, cover and allow gel to swell at least 3 hours,
preferably overnight.
- 6) After swelling, decant excess citrate/phosphate buffer
and fines and resuspend gel in 100 mL of fresh buffer.
- 7) Assemble gel chromatography system.
- 8) Place 10 mL of citrate/phosphate buffer in base of
10 column.
- 9) Load gel slurry into column as a continuous pour while
draining excess buffer out of the column.
- 10) After column is poured, allow gel to settle.
- 11) Pass 10 bed volumes (approximately 750 mL) of
15 citrate/phosphate buffer through the column and seal
for use the next day. (Add 0.01% sodium azide to
elution buffer if storing the column for more than 24
hrs).
- 12) The next day, drain excess buffer from the top of the
20 column, leaving 5 mL above gel.
- 13) Prepare 0.1 M phosphate buffer, pH 7.5, by mixing
1.361 g anhydrous potassium phosphate, monobasic
(KH_2PO_4), in 90 mL of deionized water, titrate to
pH 7.5, transfer to a 100 mL volumetric flask and fill
25 to the mark with deionized water.

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- 1 14) Prepare 0.5 M acetic acid by diluting 2.0 mL of concentrated acetic acid (17.4 M) with 67.6 mL deionized water.
- 5 15) Prepare 6 mg N-succinimidyl pyridyl dithiopropionate in 1.0 mL 30% ethanol.
- 16) Weigh 165 mg sodium acetate, combine with 10 mL deionized water and titrate to pH 5.5.
- 17) Weigh 95 mg dithiothreitol and dissolve in 250 microliters of sodium acetate buffer in a microcentrifuge tube.
- 10 18) Dissolve IgG antibody (e.g. 25 mg) in 0.1 M phosphate buffer (pH 7.5) at a concentration of 5 mg/mL.
- 19) Add 150 microliters of N-succinimidyl pyridyl dithiopropionate in 30% ethanol while stirring slowly, and continue stirring for 30 minutes.
- 15 20) Slowly decrease the pH (dropwise) of the antibody solution to pH 5.5 with 0.5 M acetic acid.
- 21) Add 50 microliters of dithiothreitol, cover solution with nitrogen and allow to stand 1 hour.
- 20 22) Drop the buffer level over SEPHADEX (Pharmacia) column to top of column and slowly add IgG mixture.
- 23) Run IgG into column and wash in with 2-3 mL citrate/phosphate buffer.
- 25 24) Fill column to top with buffer previously purged of oxygen with nitrogen, careful not to disturb column, and attach buffer reservoir layered with nitrogen or

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- 1 . argon or continuously bubble nitrogen through the
 elution buffer.
- 25) Elute column at 0.5 to 1.0 mL/min.
- 26) Layer each fraction with nitrogen or argon to avoid
5 oxidation of sulfhydryl groups.
- 27) Assay fraction on spectrophotometer at 280 nm and note
 protein peaks. Usually, two peaks are detected but
 the first protein peak is used. The second peak is
 probably immunoglobulin fragments, though no
10 confirmatory electrophoresis has been performed.
- 28) Combine fractions of high optical density and conduct
 a BCA protein assay according to manufacturer's
 instructions to estimate protein concentration.
- 29) If the protein concentration is too dilute, to
15 concentrate antibody under nitrogen or argon with
 Amicon ultrafiltration system according to the
 manufacturer's instructions.
- 30) Combine antibody with liposomes as previously
 discussed and proceed with conjugation.

20

C. Production of Echogenic Antibody-conjugated
Liposomes Using a Freeze-drying Methodology

This procedure describes methods and reagents required
to make acoustically reflective, oligovesicular liposomes
25 conjugated to IgG antibodies through a disulfide bridge

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- 1 using N-succinimidyl pyridyl dithiopropionate derivatives of phosphatidylethanolamine and antibody.

Reagents:

- 5 1) SPDP derivatized L- α -phosphatidylethanolamine (PE-PDP)
- 2) Phosphatidylcholine (PC)
- 3) Cholesterol (Chol)
- 4) Phosphatidylglycerol (PG)
- 5) Deionized water
- 10 6) Mannitol
- 7) Sodium citrate
- 8) Sodium phosphate (dibasic)
- 9) Sodium chloride
- 10) Reagent grade alcohol
- 15 11) Biogel A-5M (100-200 Mesh)

Equipment:

- 1) Rotary evaporator with external cooling, vacuum and heating
- 20 2) Probe sonicator and ear protection
- 3) Submicron particle size analyzer (e.g. NICOMP, Malvern, Coulter) and tubes
- 4) Fraction collector or test tube rack
- 5) Volumetric flasks
- 25 6) Stirrer/heating plate and stir bars

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- 1 7) Electronic balance (accurate to 0.1 or 0.01 mg)
- 8) Liquid scintillation vial or suitable substitute
- 9) Graduated cylinders
- 10) Repipets/pipets
- 5 11) Ring stand and clamps
- 12) NUCLEOPORE (Costar Co.) filters and membranes from 2 to 0.22 microns

Procedure:

- 10 1) Warm vials of phosphatidylcholine to room temperature.
- 2) Weigh 25 mg of cholesterol.
- 3) Dissolve cholesterol in 2.5 mL of chloroform.
- 4) Transfer to a rotoevaporator by pipet 73 mg of phosphatidylcholine, 11 mg derivatized
- 15 phosphatidylethanolamine, 16 mg cholesterol. Adjust as required for scale-up.
- 5) Attach flask to 0 degree centigrade pre-cooled rotoevaporator with 50 degree centigrade warming bath.
- 6) Dry lipid film under vacuum at rotational speed of
- 20 approximately 210 rpm.
- 7) Remove flask from rotary evaporator and place in desiccator under vacuum and cover desiccator with a dark cloth.
- 8) Dry film for 2 days.
- 25 9) Prepare 100 mM mannitol solution in deionized water.
- 10) Add 10 mL of mannitol solution per 100 mg of lipid.

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- 1 11) Rotate the flask at 210 rpm to resuspend lipid film.
- 12) When lipid film is resuspended, add one to two drops to NICOHP tube and dilute with 100 mM sugar water solution.
- 5 13) Analyze particle size.
- 14) Transfer liposomes from round bottomed flask to liquid scintillation vial.
- 15) Secure vial to ring stand and submerge vial approximately two-thirds into cool tap water flask to
10 dissipate excess heat during sonication.
- 16) Position sonicator probe about one quarter inch above the flask bottom and not in contact with the glass vial.
- 17) Set the sonicator for 10% duty and 10% power and
15 sonicate for 3 minutes.
- 18) Recheck particle size. Continue to reduce particle size using 1 minute sonication bursts at same power and with increasing duty levels until vesicles are less than 400 nm. Increase power and repeat duty
20 changes starting at 10% if additional sonication is required.
- 19) Derivatize antibody as previously described.
- 20) To conjugate reduced antibody to liposomes, combine liposomes with reduced, derivatized protein to have a

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- 1 coupling ratio (μg protein/ μmol lipid ---.6 mg
protein/ μmol lipid) of 390-500 and a final protein
concentration of approximately 0.5 mg/mL. Buffer
associated with protein should provide sufficient
5 buffering capacity to maintain pH at 7.0 at a minimal
ionic strength.
- 21) Allow antibody conjugation to proceed for 12-18 hours
at room temperature.
- 22) Fill chromatography column with gel at a ratio of at
10 least 10 mL of BIOGEL-A 5M (100-200 mesh) to 1 mL of
liposome suspension. Put 10 mL of buffer in column
before adding gel and then fill the column while
slowly running out the excess buffer.
- 23) Elute Biogel-A-5m column with 10 bed volumes of 100 mM
15 mannitol solution the day before use.
- 24) After antibody conjugation, pass liposomes through
sequentially smaller (2 to .22 micron) NUCLEPORE
(Costar Co.) polycarbonate filters as required to
reduce particle size below 400 nm. Verify
20 intermediate and final particle size.
- 25) Drain excess buffer from column.
- 26) Gently and slowly load liposome suspension to top of
column with a Pasteur pipet.
- 27) Slowly run liposomes into column, stopping the flow
25 when the liposomes have just completely entered the
top of the column. Do not allow the column to dry.

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- 1 28) Wash liposomes off the sides of column with 2-3 mL of mannitol solution then run this into the column.
- 29) Slowly fill the column with 100mM mannitol solution containing 0.01 M potassium phosphate buffer, pH 7.0
5 being careful not to disturb the top of the gel column.
- 30) When the column is full of buffer, attach the buffer reservoir to the column with tubing.
- 31) Attach column to fraction collector with sufficient
10 tubes to collect 3 bed volumes. Liposomes will come out in the void volume and unbound antibody will follow close behind.
- 32) Collect fractions at a rate of 0.5 - 1.0 mL/min for best resolution.
- 15 33) Analyze fractions for particles using the NICOMP at maximum photopulse to isolate liposomes and a spectrophotometer at 280 nm to isolate protein fractions.
- 34) Pool fractions containing both liposomes and protein.
- 20 35) Concentrate liposomes to approximately 10 mg of lipid per mL using an appropriately sized Amicon ultrafiltration system (300 KDa membranes) according to the manufacturer's instructions.
- 36) Transfer 5 mL aliquots of liposomes into 25 mL liquid
25 scintillation vials, layer with nitrogen and cap.

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- 1 37) Either snap-freeze in acetone-alcohol-dry ice bath or
freeze overnight in -70 degree centigrade deep
freezer.
- 38) Transfer vials from the freezer to the lyophilizer
- 5 jars and freeze dry for 48 hours according to the
manufacturer's instructions.
- 39) After 48 hours of lyophilization, remove vials from
the freeze dryer, gently layer contents with nitrogen,
recap and seal with parafilm.
- 10 40) Store vials in refrigerator with desiccant until use
within 72 hours.
- 41) Prepare 0.10 M Tris HCl + 0.10 M NaCl, pH 7.5, buffer.
- 42) To rehydrate, add 0.10 mM Tris HCl + 0.10 mM NaCl, pH
7.5, buffer to each vial. The initial liposome size
- 15 will vary inversely with the volume of buffer added.
A good starting volume is 2-3 mL of buffer/mL
lyophilized liposome containing sufficient amount of
ions to make a solution of the same osmolarity/mL of
liposome suspension lyophilized.
- 20 43) Size liposomes with submicron particle analyzer.
- 44) Transfer liposome suspension to an appropriately sized
syringe and extrude liposomes to desired particle size
through polycarbonate membranes according to
manufacturer's instruction until desired size is
- 25 attained. Note: Excessive extrusion will

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1 significantly destroy liposome structure and diminish
echogenicity. Be sure sample dilution buffer for
particle size analysis is iso-osmotic with liposome
solution to avoid artifactual swelling or contraction
5 of vesicles and inaccurate size estimation.

45) Transfer liposomes into small 10 mL liquid
scintillation vials and image liposome suspension with
20 MHz CVIS intravascular imaging catheter system.

10 EXAMPLE 3

Liposomes of four different compositions were prepared
in triplicate using the procedures outlined in Example 1
(without the optional addition of mannitol cryoprotectant)
15 and imaged with a 20 MHz intravascular catheter to
identify echogenicity. Two replicates were ultrasonically
analyzed pre and post extrusion and the results of an
overall split-plot statistical analysis $y = \text{rep} +$
 $\text{composition} + \text{rep} * \text{composition} + \text{time} + \text{composition} * \text{time} +$
20 residual , where $\text{rep} * \text{composition}$ was used to test
composition effects and residual error was used to test
time effects (time equating to pre versus post

25

1 polycarbonate extrusion). Figures 1 through 8 are examples from the second replicate pre and post polycarbonate extrusion. Least-square means for the composition effect for the above model are presented.

	<u>Composition</u>	<u>Liposome</u> <u>Mean Gray Scale</u>	<u>Liposome</u> <u>Gray Scale</u> <u>Heterogeneity</u>
	PC	3.31	3.34
10	PC:CH	9.82	8.33
	PC:PE	21.68*	11.94*
	PC:PE:CH	23.85*	13.86*

***PC vs other combination (p ≤ 0.05)**

In this study, change in solution acoustic reflectivity was quantitated as the increased pixel brightness (gray scale) of liposome clusters compared with background levels and the increase in pixel heterogeneity of the overall liposome/buffer image. Liposomes incorporating phosphatidylethanolamine were ultrasonically visible before and after extrusion to reduced particle sizes and this was reflected as a statistically significant increase in pixel gray scale and heterogeneity.

25 Phosphatidylcholine: cholesterol vesicles were echogenic before but not after extrusion.

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1 Although the invention has been described primarily in
connection with special and preferred embodiments, it will
be understood that it is capable of modification without
departing from the scope of the invention. The following
5 claims are intended to cover all variations, uses, or
adaptations of the invention, following, in general, the
principles thereof and including such departures from the
present disclosure as come within known or customary
practice in the field to which the invention pertains, or
10 as are obvious to persons skilled in the field.

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WE CLAIM:

1. A composition comprising: acoustically reflective oligolamellar liposome containing internally separated lipid bilayers.

2. The composition of Claim 1 wherein said liposome contains charged lipids.

3. The composition of Claim 1 wherein said liposome contains phosphatidylethanolamine.

4. The composition of Claim 1 wherein a ligand is conjugated to said lipid bilayer to increase separation of said bilayers.

5. The composition of Claim 1 wherein said liposome is conjugated to a tissue specific ligand.

6. The composition of Claim 5 wherein said ligand is an antibody.

7. The composition of Claim 1 wherein said liposome is between about 0.8 and 10 microns in diameter.

8. The composition of Claim 1 wherein said liposome contains a contrast agent.

9. A composition comprising: acoustically reflective oligolamellar liposome containing internally separated lipid bilayers, said liposome consisting essentially of phosphatidylethanolamine and other phospholipids.

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10. The composition of Claim 9 wherein said phospholipids are phosphatidylcholine and phosphatidylethanolamine.

11. The composition of Claim 9 wherein said liposome includes cholesterol.

12. The composition of Claim 9 wherein said liposome is conjugated to a tissue specific ligand.

13. The composition of Claim 12 wherein said ligand is an antibody.

14. The composition of Claim 9 wherein said liposome is between about 0.8 and 10 microns in diameter.

15. The composition of Claim 9 wherein said liposome contains a contrast agent.

16. An acoustically reflective oligolamellar liposome containing internally separated lipid bilayers made by the process comprising:

- a) combining lipids including phosphatidylethanolamine to form a liposome particle;
- b) reducing the size of said particle to less than about 400 nm;
- c) lyophilizing said particle; and
- d) reducing particle size to between about 0.8 and 10 microns in diameter.

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17. The composition of Claim 16 wherein said liposome is functionalized to receive a tissue specific ligand.

18. The composition of Claim 17 wherein said ligand is an antibody.

19. An acoustically reflective oligolamellar liposome containing internally separated lipid bilayers made by the process comprising:

- a) combining lipids, including phospholipids, in an organic solvent to form a solution;
- b) agitating said first solution to form a water-in-oil emulsion;
- c) combining said emulsion with a sucrose solution to form an emulsion solution;
- d) agitating said second solution to form a water-in-oil-in water emulsion;
- e) evaporating said organic solvent to form a multicompartmental liposome; and
- f) reducing the size of the liposome to between about 0.8 and 10 microns in diameter.

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20. An acoustically reflective oligolamellar liposome containing internally separated lipid bilayers made by the process comprising:

- a) combining like charged phospholipids to form a multilamellar liposome;
- b) repeatedly freezing and thawing said liposome to form multicompartamental vesicles; and
- c) reducing the size of said liposome to between about 0.8 and 10 microns in diameter.

21. An acoustically reflective oligolamellar liposome containing internally separated bilayers made by the process comprising:

- a) preparing N-succinimidylpyrolyl-dithiopropionate derivatives of phosphatidylethanolamine;
- b) combining step (a) with components selected from the group consisting of phosphatidylcholine, cholesterol and phosphatidylglycerol, to form a liposome;
- c) reducing said liposome to less than about 400 nm;
- d) conjugating an antibody to the N-hydroxysuccinimidyl-dithiopropionate derivatives of phosphatidylethanolamine;

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- e) collecting antibody-liposome complex;
- f) separating free antibody from antibody-liposome complexes;
- g) lyophilizing said conjugated liposomes;
- h) reducing particle size to between about 0.8 and 2.0 microns in diameter.

22. A method to make acoustically reflective oligolamellar liposomes containing internally separated bilayers comprising the steps of:

- a) combining phosphatidylethanolamine with other lipids to form a liposome particle;
- b) reducing the size of said particle to less than about 400 nm;
- c) lyophilizing said particle; and
- d) reducing particle size to between about 0.8 and 10 microns in diameter.

23. The method of Claim 22 wherein said liposome is functionalized to receive a tissue-specific ligand.

24. The method of Claim 23 wherein said ligand is an antibody.

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25. A method to make acoustically reflective oligolamellar liposomes containing internally separated lipid bilayers comprising the steps of:

- a) combining lipids, including phospholipids in an organic solvent to form a solution;
- b) agitating said solution to form a water-in-oil emulsion;
- c) combining said emulsion with a sucrose solution to form a second solution;
- d) agitating said second solution to form a water-in-oil water emulsion;
- e) evaporating said organic solvent to form a multicompartamental liposome; and
- f) reducing the size of the liposome to between about 0.8 and 10 microns in diameter.

26. A method to make acoustically reflective oligolamellar liposome containing internally separated lipid bilayers comprising the steps of:

- a) combining phospholipids to form a multilamellar liposome;
- b) repeatedly freezing and thawing said liposome to form multicompartamental vesicles; and
- c) reducing the size of said liposome to between about 0.8 and 10 microns in diameter.

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27. A method to make acoustically reflective oligolamellar liposome containing internally separated lipid bilayers comprising the steps of:

- a) preparing N-succinimidylpyridyl-dithiopropionate derivatives of phosphatidylethanolamine;
- b) combining step (a) with components selected from the group consisting of phosphatidylcholine, cholesterol and phosphatidglycerol, to form a liposome;
- c) reducing said liposome to less than about 400 nm;
- d) conjugating an antibody to the N-succinimidylpyridyl-dithiopropionate derivatives of phosphatidylethanolamine;
- e) collecting antibody-liposome complexes;
- f) separating free antibody from antibody-liposome complexes;
- g) lyophilizing said conjugated liposomes;
- h) reducing particle size to between about 0.8 to 2.0 microns in diameter.

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28. A method to characterize specific tissue comprising:
- a) administering acoustically reflective oligolamellar liposomes containing internally separated lipid bilayers;
 - b) measuring ultrasonic reflectance of said specific tissue; and
 - c) comparing said measured reflectance pre and post administrants to characterize said specific tissue.
29. A method to characterize specific tissue comprising:
- a) administering an acoustically reflective oligolamellar liposome containing internally separated lipid bilayers, said liposomes being conjugated to a tissue-specific ligand;
 - b) measuring ultrasonic reflectance of said specific tissue; and
 - c) comparing said measured reflectance to a control to characterize said specific tissue.
30. The method of Claim 29 wherein said ligand is a monoclonal antibody.

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31. A method to characterize thrombus comprising:
 - a) administering acoustically reflective oligolamellar liposomes containing internally separated lipid bilayers conjugated to anti-fibrin antibody;
 - b) measuring ultrasonic reflectance;
 - c) comparing said measured reflectance to a control to characterize said thrombus;
 - d) lyophilizing said conjugated liposomes; and
 - e) reducing particle size to between about 0.8 to 2.0 microns in diameter.
32. A method to monitor drug delivered in a liposome administered to a patient's circulatory system comprising:
 - a) mixing a liposome containing the drug targeted to a specific tissue and an acoustically reflective oligolamellar liposome targeted to the same tissue to form a dispersion;
 - b) administering said dispersion to said patient and monitoring the delivery of said drug from said liposome containing said drug by detecting said acoustically liposome.

FIG. 1

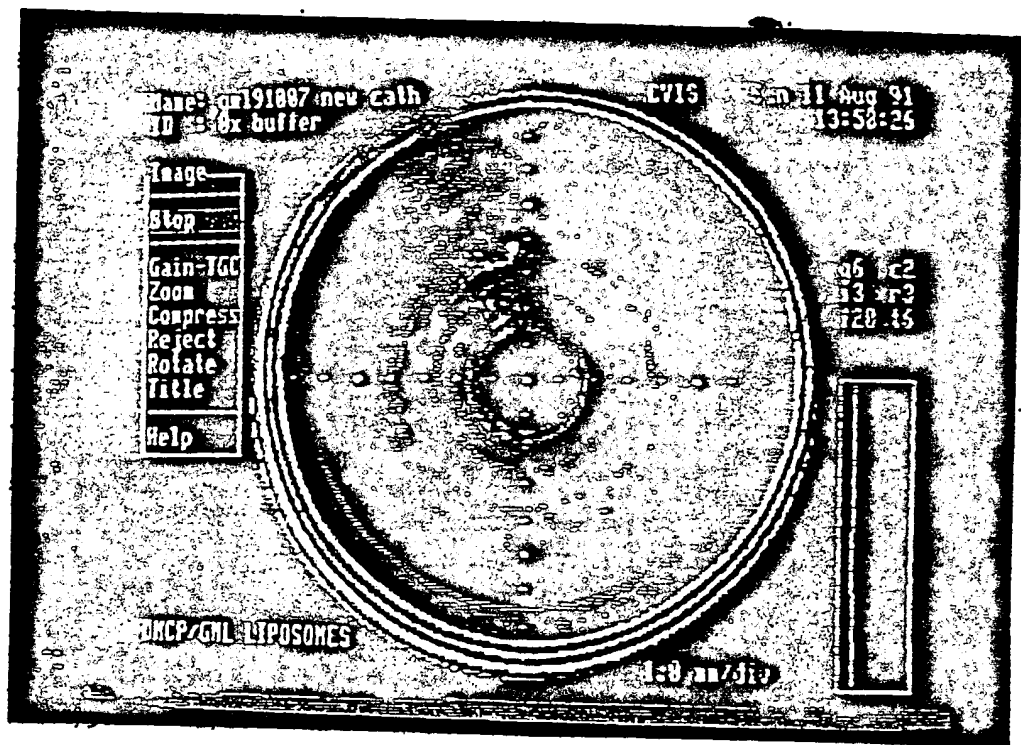


FIG. 2

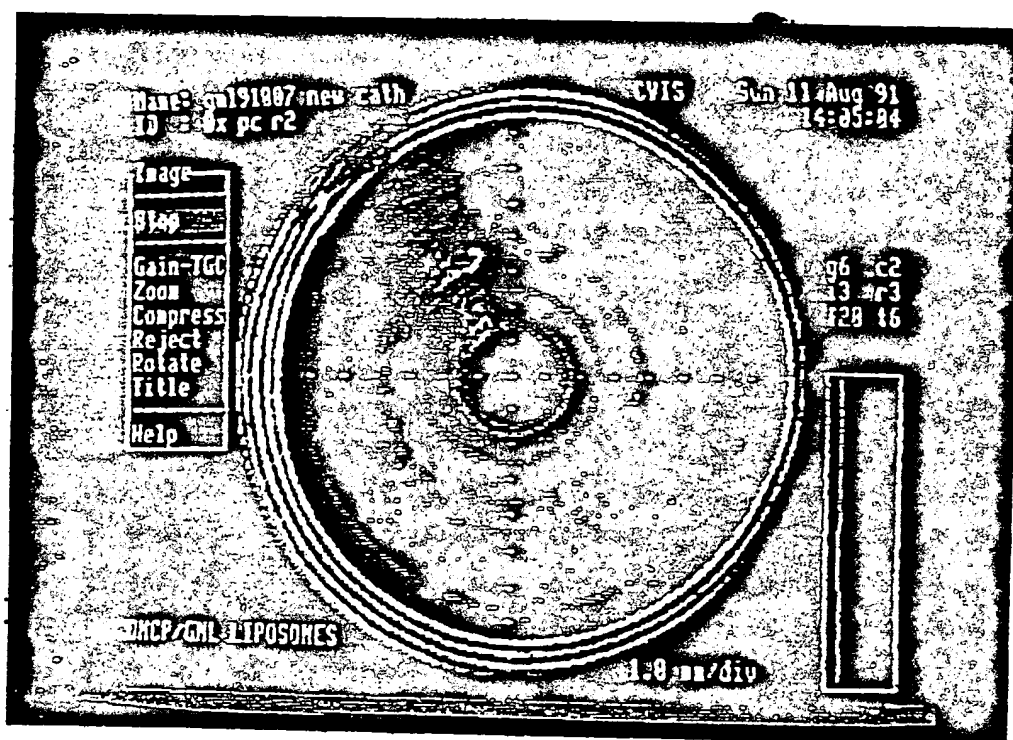


FIG. 3

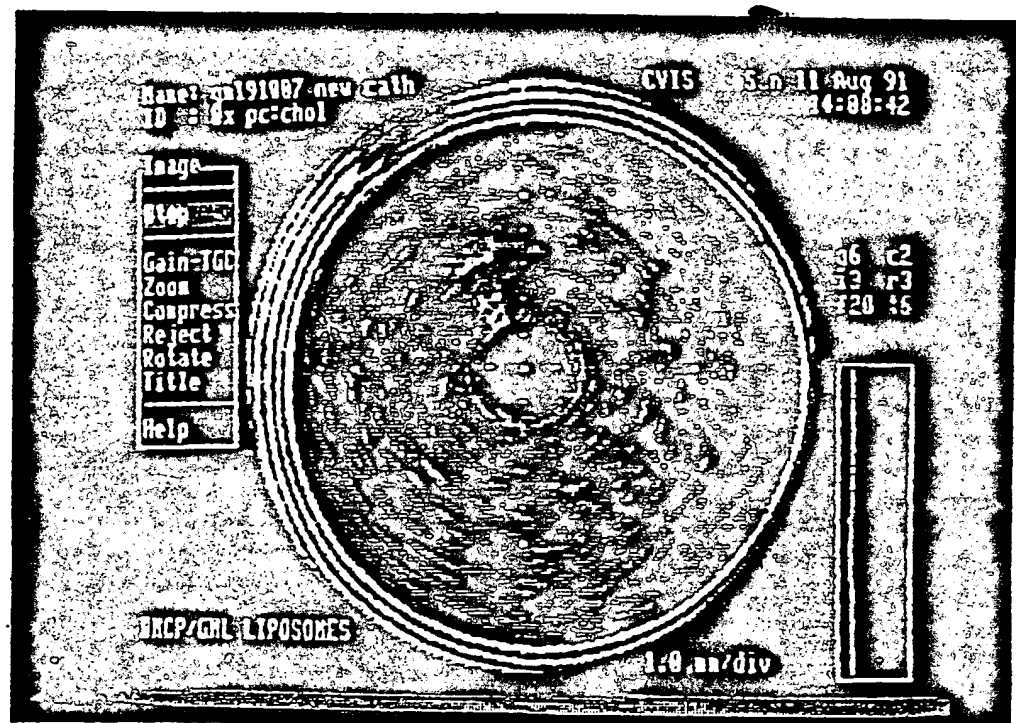


FIG. 4

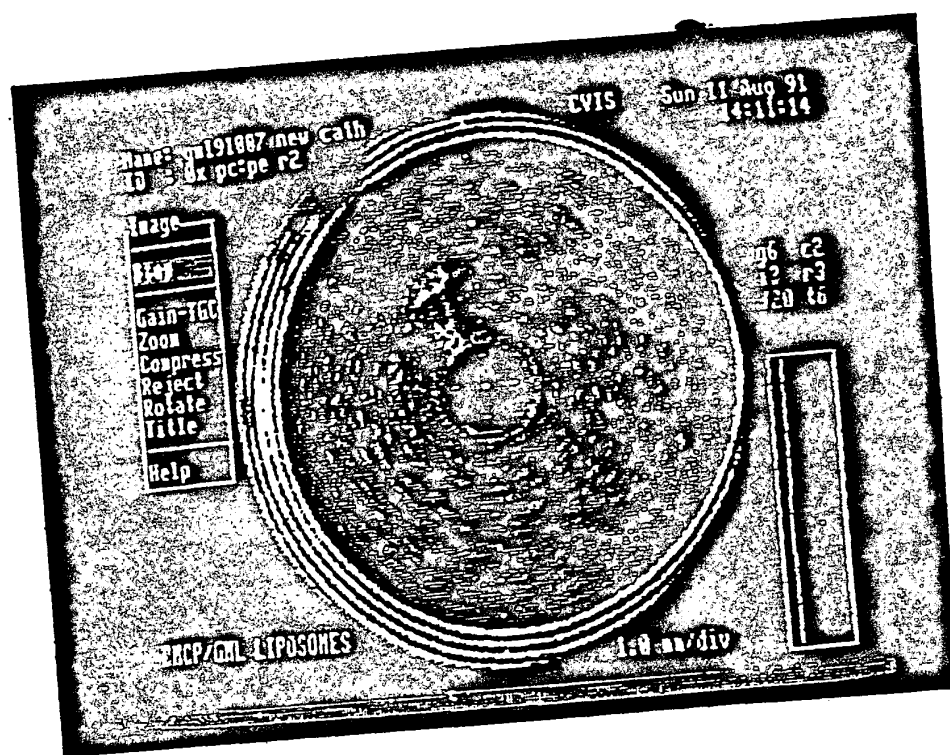


FIG. 5

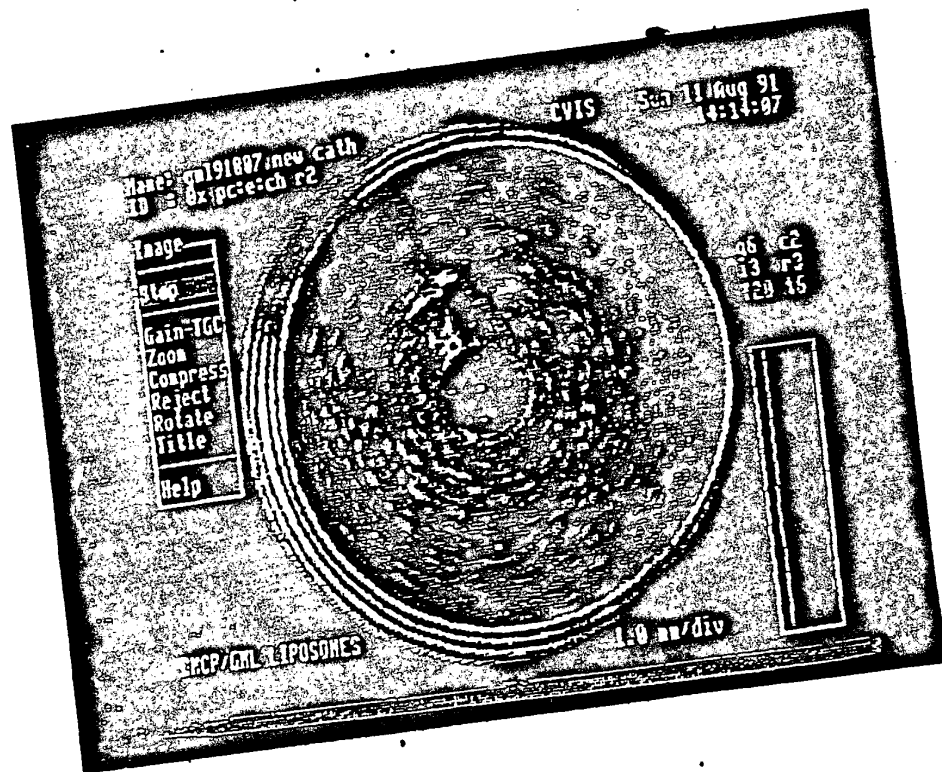


FIG. 6

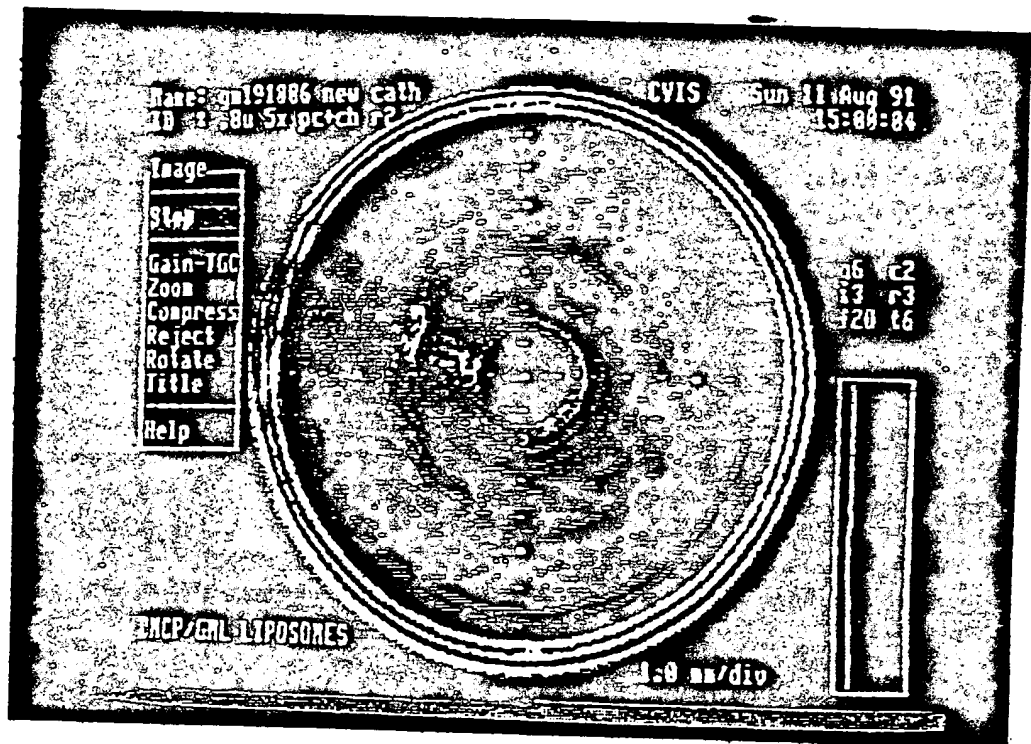


FIG. 7

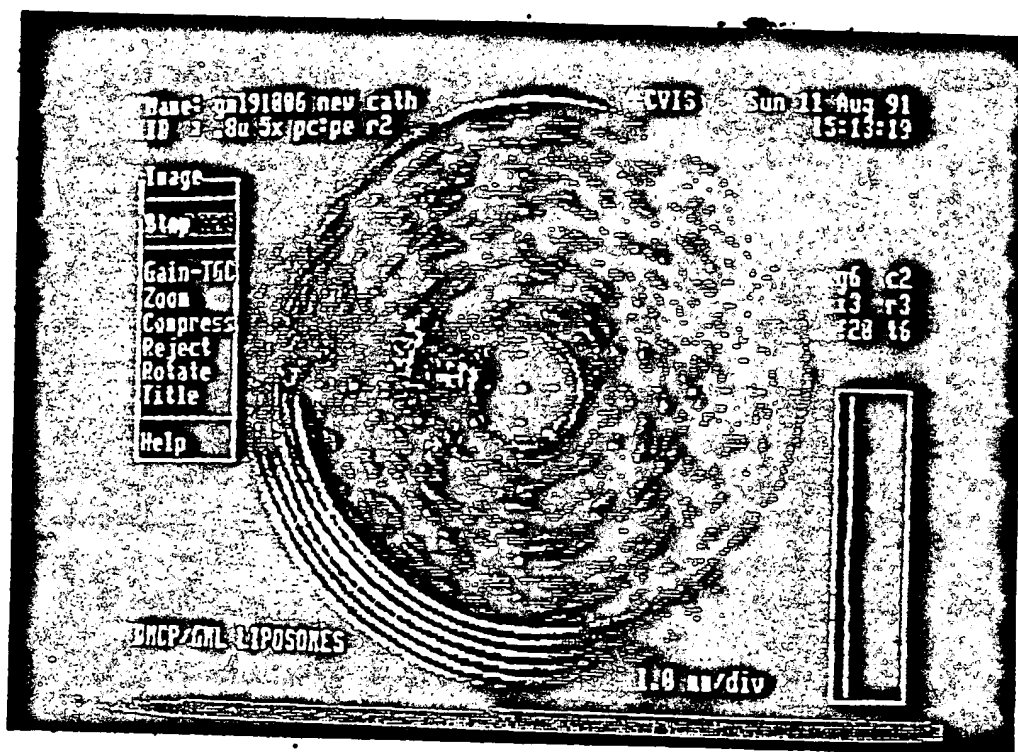
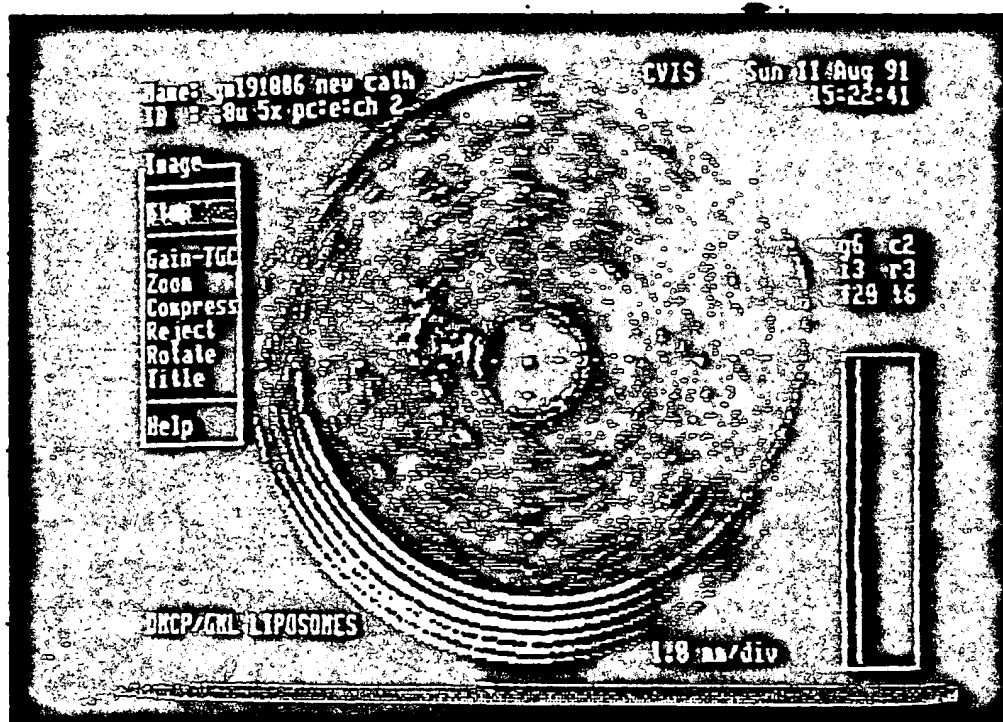


FIG. 8



PHOSPHOLIPID
BILAYER

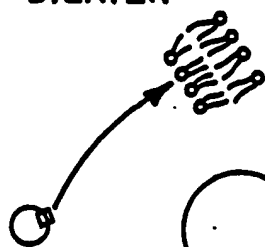


Fig. 9A

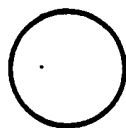


Fig. 9B

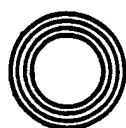


Fig. 9C



Fig. 9D



Fig. 9E

A. CLASSIFICATION OF SUBJECT MATTER

IPC(S) :A61K 9/127

US CL :424/450

According to International Patent Classification (IPC), or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/450, 4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 4,873,035 (WONG) 10 OCTOBER 1989, See entire document.	1-24, 28-30
A	US, A, 4,900,549 (DE VRIES ET AL) 13 FEBRUARY 1990, See entire document.	1-24, 28-30
A	US, A, 4,971,916 (JOU ET AL) 20 NOVEMBER 1990, See entire document.	1-24, 28-30

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents	*T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principles or theory underlying the invention
A document defining the general state of the art which is not considered to be part of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

12 AUGUST 1993

Date of mailing of the international search report

09 SEP 1993

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Authorized officer

G. S. KISHORE

Facsimile No. NOT APPLICABLE

Telephone No. (703) 308-2351

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
(Form PCT/ISA/206 Previously Mailed.)
Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-24 and 28-30

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Species in method of making claims:

- a) Method in claims 22-24
- b) Method in claim 25
- c) Method in claim 26
- d) Method in claim 27

II. Species in method of use claims:

- a) Method of use of claims 28-30
- b) Method in claim 31
- c) Method in claim 32

Species I, a-d are independent and distinct in that they are different methods of making the composition.

Species II, a-c are different methods of use and thus are patentably distinct